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Early reduction of circulating homocysteine levels in Goto–Kakizaki rat, a spontaneous nonobese model of type 2 diabetes

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ABSTRACT

Diabetes mellitus is associated with increased risk for cardiovascular disorders, which are major causes of mortality in this disease. Hyperhomocysteinemia, defined by high plasma homocysteine levels, is an independent risk factor for the development of cardiovascular diseases. Type 2 diabetic patients have higher circulating homocysteine levels than healthy subjects and these levels are even higher in plasma of obese than nonobese diabetic patients. Homocysteine metabolism that has been studied in 2 animal models of type 2 diabetes with obesity led to conflicting data. The aim of the present study was to analyze homocysteine metabolism in a spontaneous nonobese model of type 2 diabetes, the Goto–Kakizaki rats at various successive and well characterized stages of the disease: during early postnatal normoglycemia, at the onset of hyperglycemia (around weaning), and during chronic mild hyperglycemia with progressive insulin resistance. Compared to age-matched Wistar controls, Goto–Kakizaki rats showed lower plasma levels of homocysteine and a falling trend in its major byproduct antioxidant, glutathione, from the prediabetic stage onwards. Concomitantly, Goto–Kakizaki rats exhibited increased liver activity of cystathionine beta synthase, which catalyzes the condensation of homocysteine with serine in the first step of the transsulfuration pathway. These results emphasize a strong association between homocysteine metabolism and insulin via the first step of the hepatic transsulfuration pathway in Goto–Kakizaki rats.

1. Introduction

Cardiovascular diseases are the major cause of death in diabetic patients [1]. Hyperhomocysteinemia, defined by high plasma homocysteine (Hcy) levels, is now well recognized as an independent risk factor for the development of cardiovascular diseases [2]. Hcy, an intermediate in the sulfur amino acid metabolism [3], is metabolized by remethylation to methionine or transsulfuration to cysteine. Cystathionine beta synthase (CBS), the first enzyme involved in the transsulfuration pathway, catalyzes the condensation of Hcy with serine to form cystathionine. Cystathionine is subsequently hydrolyzed to form cysteine and cysteine, in turn, can be incorporated into protein, or used to synthesize the antioxidant glutathione (GSH) via the γ-glutamyl cycle. GHS is either used as cellular antioxidant or exported and becomes a substrate for γ-glutamyl transpeptidase. The action of γ-glutamyl transpeptidase leads to the formation of cysteinylglycine, which is subsequently cleaved by dipeptidase to form cysteine and glycine. Cysteine can then be transported into the cell and thus be again used for GSH synthesis.

The relationship between diabetes and plasma Hcy levels has been studied in both humans and rodents. Plasma Hcy levels have been found to be higher in type 2 diabetic patients than in healthy subjects and also, among type 2 diabetic patients, in obese than in nonobese individuals [4]. By contrast, reduced serum Hcy levels were described in type 2 diabetic patients without cardiovascular complications or diabetic nephropathy [5]. Chronic hyperglycemia was thus suggested to affect its renal excretion, or accelerate hepatic transsulfuration secondary to insulin disorders [5]. Moreover, subjects with insulin resistance had significantly lower serum Hcy levels compared with non-insulin resistant subjects [6]. Animal models of type 2 diabetes were then used to better understand the relationship between insulin resistance and/or hyperglycemia and circulating Hcy levels. In long-term fed rats with a high-fat-sucrose (HFS) diet, which induces hyperinsulinemia,

Abbreviations: CBS, cystathionine beta synthase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); GK, Goto–Kakizaki; GSH, glutathione; Hcy, homocysteine; HFS, high-fat-sucrose; STZ, streptozotocin; tHcy, total Hcy; ZDF, Zucker diabetic fatty

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insulin resistance and obesity but not hyperglycemia, plasma Hcy levels were higher than in control rats fed at low-fat, low-carbohydrate diet [7]. The Zucker diabetic fatty (ZDF) rat is a spontaneous type 2 diabetic obese animal model characterized by a prediabetic insulin-resistant stage followed by a rapid transition to frank hyperglycemic stage due to relative hypoinsulinemia. In the ZDF rat, plasma Hcy levels were reduced at both prediabetic and diabetic stages, compared to respective control groups [8]. Thus, conflicting data were observed in 2 rat models of type 2 diabetes with obesity.

The Goto–Kakizaki (GK) rat is another spontaneous and well characterized but nonobese model of type 2 diabetes [9]. In this model, a normoglycemic period, which associates low insulinemia but elevated whole body insulin sensitivity precedes onset of hyperglycemia around weaning [10]. The aim of the present study was to analyze Hcy metabolism at 4 crucial steps of the GK rat life: in normoglycemic 2-week-old neonates and at 1 month of age (onset of hyperglycemia), and 2 of 3 months of age (aggravation of insulin resistance) [11].

2. Materials and methods

2.1. Rats

All animal care was conducted in accordance with internal guidelines of the French Agriculture Ministry for animal handling. Rats were housed in a controlled environment with unlimited access to food and water on a 12-h light/dark cycle. Number of rats and suffering were minimized as much as possible. All animal experiments were conducted on age-matched male Goto–Kakizaki (GK) and nondiabetic (control) Wistar rats from our laboratory [11]. The GK line was established by repeated inbreeding from Wistar rats selected at the upper limit of normal distribution for glucose tolerance [9,11].

2.2. Blood, tissue collection and assays

Male rats were weighed, killed by decapitation and blood samples were collected and placed on ice immediately. Basal fed morning glycemia was determined with a glucometer. Plasma was isolated by centrifugation at 2500*g* for 15 min at 4 °C. Livers were harvested, snap-frozen and stored at −80 °C until use. Plasma total Hcy (tHcy), defined as the total concentration of Hcy after quantitative reductive cleavage of all disulfide bonds, and total glutathione (GSH) were assayed by using the fluorimetric high-performance liquid chromatography method as previously described [12]. The inter- and intra-assay coefficients of variation for mean tHcy level were 4.2% and 6.3%, respectively, and the linearity was from 1 to 100 μM [13]. Serum insulin was assayed by ELISA (Rat Insulin Elisa, cat. 10-1124-01, Mercodia, Uppsala, Sweden) [14].

2.3. CBS enzyme activity assays

Determination of CBS activity was assayed on 400 μg of total proteins obtained from liver samples, as described [15]. Proteins were incubated for 1 h at 37 °C with 1 mM of propargylglycine, 0.2 mM of pyridoxal phosphate, 10 mM of L-serine, 10 mM of DL-Hcy, 0.8 mM of SAM, using DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) based-assay. All the chemical products were obtained from Sigma (Sigma-Aldrich, France).

2.4. Data analysis

Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t*-test using Statview software. In both cases, Student–Newman–Keuls tests were used for multiple pairwise comparisons. The results are expressed as mean ± SD. Data were considered significant when $p<0.05$.

3. Results and discussion

3.1. Body weight and metabolic parameters in GK rats as a function of age

As previously described [10], male GK rat body weights were significantly lower compared to age-matched Wistar rats (Fig. 1A). Circulating glucose levels were similar in GK and Wistar rats at 2 weeks of age (Fig. 1B), but significantly increased in GK rats from 1 month of age (weaning) (Fig. 1B). Indeed, GK rats become hyperglycemic around weaning, when the diet shifts from lipid-enriched maternal diet to carbohydrate-enriched laboratory chow [16].

In 2-week-old GK rats, basal insulinemia was significantly decreased (Fig. 1C). However, because GK neonates exhibit a transient increase in whole body insulin sensitivity as previously described [10], they stay normoglycemic until around weaning. Then, GK rats displayed mildly elevated but not statistically significant insulin levels at 1 month of age and the hyperinsulinemia became statistically significant at 2 and 3 months of age, once insulin multi-organ resistance and especially liver insulin resistance has been established [11,17]. Thus, in addition to HFS-fed rats and ZDF rats, GK rats appeared to be a suitable but nonobese model to analyze Hcy metabolism in association with alterations of glucose metabolism.

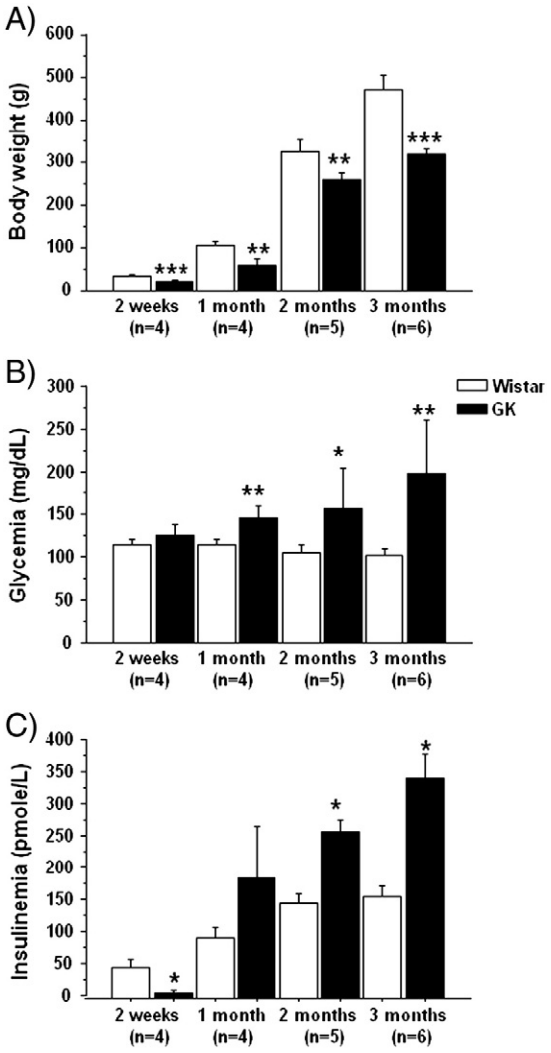

Fig. 1. Age-dependent changes in body weight, and in basal plasma glucose and insulin levels in GK and Wistar rats. Postnatal growth (A). Glucose (B) and insulin (C) levels were determined.*$p<0.05$, **$p<0.005$, ***$p<0.0005$ versus age-matched Wistar group.

3.2. Hcy metabolism in GK rats as a function of age

Fig. 2A shows that GK plasma tHcy levels were only about 45% of that found in age-matched Wistar rats in both the prediabetic and diabetic periods. These low plasma tHcy levels are in agreement with those described in both type 1 STZ-induced diabetic rat model [18] and spontaneous type 2 diabetic obese ZDF rats [8].

From the prediabetic stage onwards (Fig. 2B), GK rat also showed a falling trend in plasma GSH levels, the effect being only significant at 2 weeks and 3 months of age. GSH is the most abundant low-molecular-weight thiol and plays a key role in the cellular defense against oxidative stress. GSH is a reference of antioxidant and its antiatherogenic properties have been demonstrated in humans and rodents [19,20]. Here, it should be underlined that decreased plasma GSH levels are indicative of a compromise protection against oxidative stress, which is already present in prediabetic GK animals [21].

The hepatic activities of several enzymes involved in the remethylation and transsulfuration pathways that play a role in the removal of Hcy were found to be increased in ZDF rat liver [8]. Previous results emphasized a direct effect of insulin in repressing CBS expression in both human and rat cultured hepatocytes [22]. Therefore, to examine whether the lowering of GK rat plasma Hcy levels, reflecting cellular and mainly liver production, could result from increased Hcy catabolism through the first step of the transsulfuration pathway, we measured the CBS enzyme activity. Given that the majority of dietary methionine is metabolized in the liver, which contributes to much of the plasma Hcy levels, we analyzed the hepatic enzyme activity [23]. Fig. 2C shows that the hepatic CBS activity was significantly elevated in GK versus Wistar rats and, once again, the effect was present from the prediabetic stage onwards. Thus, low plasma tHcy levels were associated with increased hepatic CBS enzyme activity in GK rat. However, increased CBS activity does not imply increased transsulfuration flux. This flux is determined by the rate at which methionine enters into hepatic metabolism, which will be largely determined by dietary methionine consumption. In the experimental mouse, hyperhomocysteinemia is induced by methionine-enriched diet, and increased plasma Hcy levels are accompanied by proportional increased GSH levels, without modification of cysteine and cysteinylglycine levels [24]. The major effect of increased CBS activity is to decrease the steady-state Hcy concentration at which transsulfuration occurs and then contributes to the decreased plasma Hcy level. Therefore, decreased circulating GSH levels may reflect decreased circulating Hcy levels in GK rats.

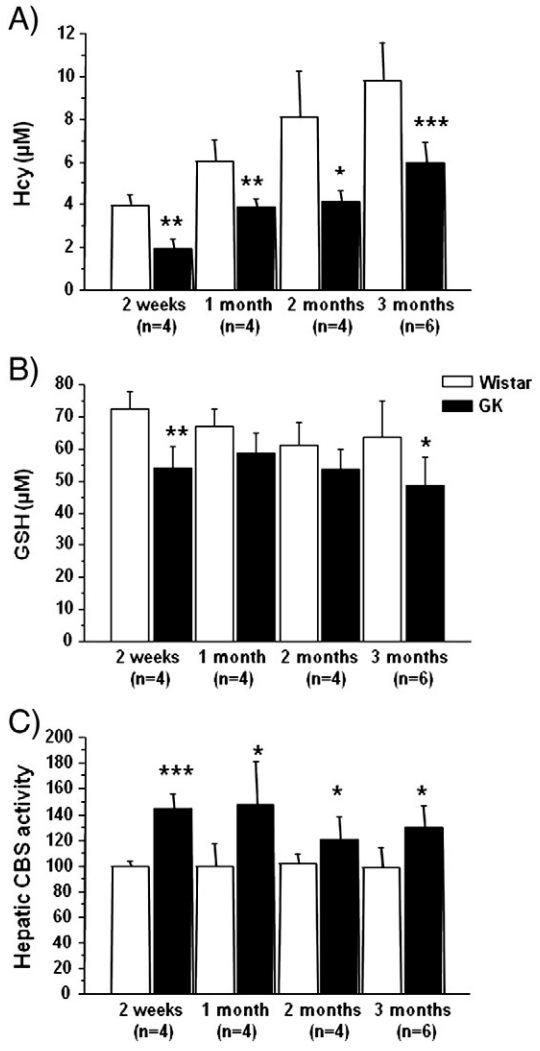

Fig. 2. Age-dependent changes in plasma Hcy and GSH levels, and relative hepatic CBS activity. Hcy (A) and GSH (B) levels were determined in plasma. (C) CBS activity assay was performed on extracts from liver of individual rats. CBS activity values are normalized from age-matched Wistar rats. $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0005$ versus age-matched Wistar group.

Similar data demonstrate low plasma tHcy levels with elevated hepatic CBS activity in 3 different rat models of diabetes, STZ-induced type 1 diabetes [18] and type 2 diabetes, either obese ZDF [8] or nonobese GK rats (the present study). Thus, the association between low plasma tHcy levels and high CBS activity does not depend on the type of diabetes and the presence of obesity and is observed during the short period of prediabetes in both type 2 models.

The repression of hepatic CBS expression and activity by insulin has been demonstrated in STZ-induced type 1 (insulin-dependent) diabetic rats [18,22]. In this model, increased hepatic CBS enzyme activity was associated with elevated CBS mRNA expression and insulin treatment normalized hepatic CBS expression and activity. Type 2 diabetes is characterized by relative lack of insulin and/or the resistance to the action of insulin. Insulin resistance is a multisite dysfunction that involves, in particular, the liver [25]. Insulin signaling in liver is critical in regulating glucose homeostasis and maintaining normal hepatic function. At 1 month of age, GK rats already exhibit increased hepatic glucose production specifically linked to early liver insulin resistance. These data highlight a possible primary role of the liver defect, and not simply a late consequence of chronic hyperglycemia [26]. Thus, the lack of functional insulin or decreased insulin sensitivity (insulin resistance) in the liver is probably at play very early, during prediabetes in both the ZDF and GK type 2 models, resulting in increased hepatic CBS activity and low plasma Hcy levels. Indeed, during the prediabetic stage, GK rats are mostly characterized by very low insulin levels, while ZDF rats already exhibit insulin resistance concomitant with hyperinsulinemia [8]. After diabetes onset, GK rats exhibit progressive hepatic insulin resistance and hyperinsulinemia [17], while ZDF rats become hypoinsulinemic in addition to being insulin resistant [8]. Therefore, the lack of insulin or the resistance to insulin action rather than hyperglycemia appear to increase hepatic CBS activity and consequently decrease plasma Hcy levels.

Because of anti-inflammatory effects of insulin [27], decreased insulin levels in GK rats from end of fetal life onwards [28] might be partly responsible for various neonatal oxidative stress-related signs, like oxidized state of GSH in red blood cells associated with low circulating GSH and elevated chemokine levels, and high islet ROS concentration and alterations in GSH- and thioredoxin-related gene expression [21]. We suggest that early insulin deficit may cause systemic and tissue oxidative stress in GK rats. This hypothesis is in agreement with 1) the situation of prediabetic insulin-resistant (i.e., with a relative lack of insulin) ZDF rats and 2) the 'unexpected' inverse relationship between insulin resistance and serum Hcy in healthy subjects [6]. Thus, hypohomocysteinemia associated with low

systemic levels of GSH (the latter has antiatherogenic properties in humans and rodents [19,20]) rather than hyperhomocysteinemia might be a risk factor for CVD in insulin resistance and diabetes in humans and rodents.

By contrast, hyperhomocysteinemia in type 1 and type 2 diabetic patients appears to be dependent on the presence of nephropathy [29,30]. GK rat does not spontaneously develop kidney disease. However, secondary injurious mechanisms such as hypertension induces progressive nephropathy [31]. By contrast, long-term (from 6 months to 2 years) HFS-fed rats, which are obese, normoglycemic, hyperinsulinemic, and insulin resistant, display hyperhomocysteinemia and lower hepatic CBS enzyme activity and mRNA expression than normally fed rats [7]. These HFS-fed rats exhibit hypertension, and their hyperhomocysteinemia probably result from kidney alterations which develop rapidly after a few weeks of diet [32,33]. Altered CBS activity in HFS-fed rat kidney may cause hyperhomocysteinemia and diminish hepatic CBS activity [34]. Accordingly, hyperhomocysteinemia in obese and/or diabetic patients might more probably reflect kidney alteration.

In conclusion, the spontaneous nonobese type 2 diabetic GK rat, characterized by an early deficit in functional beta-cell mass, shows decreased levels of circulating Hcy and a falling trend in its major byproduct antioxidant GSH from the prediabetic stage onwards. Concomitant increased hepatic CBS activity emphasizes the role of insulin in Hcy and antioxidant GSH metabolism.

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